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Vegard Sandøy Bråthen

Sammendrag

Tidlig vekst og utvikling påvirker langsiktig kroppskondisjon og overlevelse hos den langlevde sjøfuglen Krykkje (*Rissa tridactyla*). Lav mattilgang fører til økt stress hos voksne individer og kan lede til et skifte av energiforbruk mot egen overlevelse fremfor å investere i avkom. Denne studien undersøker effekten av stress hos voksne krykkjer på immunokompetanse hos avkom, ettersom det siste er anerkjent som en viktig egenskap i langsiktig overlevelse. Økt stress i foreldre ble antatt å vise en negativ innvirkning på immunokompetanse og kroppskondisjon. For å måle immunokompetanse ble forholdet mellom innate hetrofile celler og adaptive lymfocytter beregnet, sammen med en målt reaktivitet av naturlige antistoffer og komplementære molekyler på fremmed antigen. Dataene ble samlet inn over to hekkesesonger i en mellomstor koloni på vestkysten av Svalbard, Norge. I den første sesongen ble foreldrenes stressnivå manipulert ved å behandle individer med implantater fylt med eksogene stresshormoner (corticosteron) for en tre dagers periode. En kontrollgruppe ble utsatt for tomme implantater. Stress og kroppskondisjon ble målt hos foreldre og avkom, sammen med immunokompetanse i avkom, omtrent 15 dager etter implantatene ble fjernet. I den andre sesongen ble tilsvarende data innhentet uten stress-manipulasjoner. Begge sesongene hadde normale værforhold og mattilgang. Det ble sett en uventet nedgang i baseline corticosterone hos stress-manipulerte individer, med 55 % lavere stress verdier enn kontrollindivider ved måling av ungene. Individenes kroppskondisjon ble ikke påvirket av implanteringene. Dette ble fulgt av en høyere kroppskondisjon og immunokompetanse hos avkom av stress-manipulerte voksne. Mine resultater støtter en "fiksert investeringshypotese", der foreldre vil øke sin reproduktive innsats hvis stress er redusert under normale forhold. Immunokompetanse ble bare delvis påvirket av foreldrenes stressnivå, mens kroppskondisjonen var signifikant høyere hos unger av corticosteron-implanterte individer. Derfor ble det antatt en større fordeling av energi mot høyere kroppskondisjon, snarere enn mot immunokompetanse. Dette kan innebære en høyere seleksjon for kroppskondisjon under vekst og utvikling under normale forhold. Dette er den første studien som presenterer eksperimentelle data på immunokompetanse hos unger av krykkje når foreldrenes stressnivå er manipulert.

Abstract

Low food availability increase food stress and can cause a shift of energy expenditure to invest in self-survival rather than reproductive effort in breeding Black-legged kittiwakes (*Rissa tridactyla*). This affects early development fitness in offspring, which impact on their long-term survival. The present study investigates effects of stress in adult kittiwakes on immunocompetence of offspring, which is recognized as important trait in long-term survival. Increased parental stress is predicted to negatively affect immunocompetence and body condition. To assess immunocompetence, a ratio between innate hetrophils and acquired lymphocytes were calculated, together with measured reactivity of natural antibodies and complement on foreign antigen. Data was collected over two breeding seasons in a medium sized colony on the west coast of Svalbard, Norway. In one season, parental stress was manipulated by exposing individuals for implants with exogenous corticosterone for a three day period. A control group was exposed to sham implants. Stress and body condition was recorded in parents and offspring, along with immunocompetence in offspring approximately 15 days after implants were removed. In the second season, data of the same measurements were collected without stress-manipulations. Both seasons provided normal foraging conditions. I found an unexpected decrease in baseline corticosterone in stress-manipulated individuals, with corticosterone values being 55% lower than that of sham- treated individuals at the latest point of sampling. Parental body condition was not affected by treatments. This was accompanied by a greater overall fitness in the offspring of stress- manipulated adults. Hence, my results support a 'fixed investment hypothesis', in which parental birds will increase their reproductive effort if food stress is decreased under normal foraging conditions. Immunocompetence was only partly affected by parental treatment, while body condition was significantly higher in chicks of corticosterone-implanted individuals. Hence, there was predicted a greater allocation of energy towards higher body condition, rather than immunocompetence. This might imply a higher selection for body condition during development, when provided normal conditions. This is the first study to present experimental data on immunocompetence in offspring of adult kittiwakes when the parental stress level is manipulated.

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List of abbreviations

ACTH	Adrenocorticotropic hormone
AICc	Akaike Information Criterion test, corrected for a small sample size
BCI	Body condition index
CORT	Corticosterone
CRH	Corticotropin-releasing hormone
HPA axis	Hypothalamus-pituitary-adrenal axis
IgM, IgY	Immunoglobulin. IgM = naïve (NAbs). IgY = specialized (maternal)
NAb	Natural Antibodies
PBS	Dulbecos phosphate buffered saline
PCA	Principle component analysis
RBC	Red blood cells
RIA	Radioimmunoassay
RL antigen	Radio labeled antigen
SHAM	control implants, empty silastic tubes
SSD	Sexual size dimorphism

1. Introduction

1.1 Life history and trade-offs

Life history theory explains trade-offs between traits that increase the fitness of the organisms, such as their fecundity, immunocompetence, growth, metabolic rate, and survival (Stearns 2000; Ricklefs & Wikelski, 2002). When available recourses are limited either by environment, e.g. food abundance and predator threat, or by physiology (Speakman & Król, 2010), these limited resources have to be allocated to various aspects of behavior and physiology to maintain homeostasis (Ricklefs & Wikelski, 2002). Reproductive attempts are energetic bottlenecks since energy needs to be allocated to the production and care of offspring, but also to the maintenance of basic body- functions. Under deteriorating foraging conditions, parents need to spend more time away from the nest to provide both adequate food for offspring and to feed themselves (Kitaysky et al., 1999; Angelier et al., 2009). This decreases their nest attendance and can thus have negative impacts on reproduction. Seabirds meet fitness trade-offs by following a 'slow-living' life history strategy. They usually prioritize investment in self-maintenance over current reproductive effort, and in breeding seasons with low food availability, reproductive success is often decreased, rather than adult survival (Kitaysky et al., 2007; Sandvik et al., 2012). There exist two hypotheses on the reproductive investment in a slow- lived seabird like the Black-legged kittiwake (*Rissa tridactyla*; hereafter 'kittiwake'), supporting either a flexible investment strategy (the flexible parental investment hypothesis, Golet et al., 1998), in which energy expenditure is increased in bad foraging years (seen in pacific Kittiwakes), or a fixed investment strategy (Welcker et al., 2010), where breeding birds operate close to an energetic ceiling independent of resource availability, and thus compromise reproductive investment during poor foraging years (seen in Atlantic kittiwakes).

1.2 Food stress and parental care

Hormones are important mediators in response to environmental changes, which in turn can affect life-history traits (Ketterson & Nolan, 1992; Hau et al., 2010). The hypothalamus-pituitary-adrenal axis (HPA axis) is an important physiological mediator for resource allocation and thus affects physiology and behavior (Sapolsky et al., 2000; McEwan & Wingfield, 2003). The glucocorticoid hormone corticosterone (CORT) is secreted by the

adrenal gland and correlates well with environmental conditions such as food availability, also in seabirds (Kitayksy et al., 1999). Deteriorating environmental conditions trigger an increased secretion of stress hormones (Kitayksy et al., 1999; Saino et al., 2003). CORT is also recognized as a primary mediator in energy allocation (Johnstone et al. 2012). Since the hormone can readily be manipulated in wild animals with simple hormone implants, it is commonly used to experimentally investigate the effects of an environmental stressor on physiology and behavior in avian vertebrates. Here stressors are defined as any factor that disturb homeostasis, and stress as a factor that elevates the output of the HPA axis above what is required of normal physiological maintenance (McEwen & Wingfield, 2003, Johnstone et al., 2012).

Effects of elevated CORT secretion vary with time and strength. Short periods (hours to days, i.e. an acute stress response period of time) with an intermediate increase in circulating CORT might affect several processes, such as enhancing immune responses (Dhabhar & McEwen, 1997; 1999), increasing feeding effort, begging from nestlings (Kitaysky et al., 2001), or promoting increased survival when a state of fight-or-flight (emergency life history stage) is triggered at even higher CORT levels (Wingfield et al., 1998). CORT released during long-term stress (days to weeks) provides energy to more long-term vital processes and reduces the overall energy expenditure, for example through a reduction in parental effort (Kitaysky et al., 1999; McEwan & Wingfield, 2003; Johnstone et al., 2012). In summary, CORT is suggested to be a major contributor in the adjustment of reproductive effort to environmental conditions.

1.3 Nestling fitness and immunocompetence

Early development, on which parental effort can have a significant effect, impacts long-term survival and fitness in kittiwakes (Cam et al., 2003). The short period of growth is a crucial life-stage, where fundamental and vital traits of physiology and morphology develop. Immunocompetence, defined as the ability to withstand infection by pathogens and parasites, is an important investment in long term survival. A stronger immunocompetence increases the individual's ability to handle wounds, sickness and infections, but can be compromised during development if conditions are poor (Apinius, 1998a; Norris & Evans, 2000). For example chicks that hatch late often have a reduced immunocompetence and a lower survival rate (Gasparini et al., 2006).

The immune system can be divided into innate and acquired immunity. The innate immune system is a first line of defense that is predeployed and unable to 'memorize' or further adapt its response to invading pathogens (Apinius, 1998a; Kindt et al., 2007). It consists of anatomical barriers, bacteria, humoral and cellular factors, which either block or attempt to rid the body of pathogens. Humoral factors are soluble elements that can cause inflammatory responses, pathogenic destruction through lysis, or stimulate acquired immune responses. Cellular factors also promote inflammation and acquired recruitment, but also engulfment and phagocytosis of pathogens (Demas & Nelson, 2012).

Acquired immunity develops a pathogen-specific response within a few days after infection, and memorizes pathogens by antigen-antibody interactions. Main generators of an acquired response are T-lymphocytes, derived from the thymus, and B-lymphocytes, which in birds are derived from the bursa of Fabricius. Acquired immunity is again divided into humoral (antibody-mediated) and cellular (cell-mediated cytotoxic) compartments. The humoral compartment consists of soluble immunoglobulin (antibody), produced by B-lymphocytes, that detects and mark foreign antigen. The cellular compartment consists of T-lymphocytes which eliminate foreign pathogens after activation (Kindt et al., 2007).

There are several ways to quantify immunocompetence. Calculating blood content of heterophils and lymphocytes of the innate and acquired immune system, respectively, are a much applied method, which results in a H:L ratio that illustrates the balance between innate and acquired immunity. A high H:L ratio reflect a low presence of acquired immunity, by low numbers of circulating lymphocytes. However, an alteration of this ratio due to e.g. sickness may be misinterpret as a representative value for immunocompetence, and should always be taken into consideration (Norris & Evans, 2000).

Challenging techniques is another approach to measuring immunocompetence that records the ability to respond to pathogenic challenges. Hemolysis-hemagglutination assays test the level of natural antibody (NAb)-mediated complement activation, which are fundamental in both innate and acquired immunity. Natural, somewhat randomly produced, antibodies (NAbs) can be classified as innate, humoral factors that mark foreign pathogens. NAbs bound to B-lymphocytes cause proliferation, structural adaptions and increased production of specific antibodies (acquired antibodies) in the lymphocyte, that further induce activation of cytotoxic T-lymphocytes, through antigen-complement interaction. NAbs also circulate freely and can cause clumping (agglutination) of foreign pathogens and further lysis by developing

membrane attack complexes together with complement molecules (Apinius, 1998a; Lundquist et al., 2006; Kindt et al., 2007).

1.4 Development and immunocompetence

During the early chick stage, avian vertebrates are mainly protected by their innate immunity, since the acquired immune system is yet poorly developed (Apinius, 1998a; Grindstaff, 2008). Launching an immune response against invading pathogens is costly and even mild sickness might exert negative effects on growth (Rivera et al., 1998). The risk of infection and sickness during early development in birds is greatly reduced by transferring maternal antibodies to offspring through the egg (Apinus, 1998a; Grindstaff et al., 2003; Grindstaff, 2008). The pathogenic environment prior to reproduction influences the antibodies transferred to offspring, hence e.g. kittiwakes breeding in areas with high tick densities transfer higher concentrations of Lyme disease agents than kittiwakes from areas with lower tick densities (Gasparini et al., 2001). Also life history strategies can determine immunocompetence levels, since slow-living species invest more in the transfer of maternal immunity to offspring than fast-living species (Tella et al., 2002).

1.5 Cost of immune defense

The true cost of maintaining an appropriate immunocompetence has been widely debated. The immune system is a combination of several components, which differ by their reactivity, interaction, cost, and vital significance (Lochmiller & Deerenberg, 2000; Norris & Evans, 2000). An animal in allostasis might involve the immunocompetence in a trade-off to achieve homeostasis, by allocating energy away from immunocompetence towards other traits that may increase fitness. Several studies claim that the immune system is subject to trade-offs in stressful situations, such as food shortages (Saino et al., 1997; Alonso-Alvarez & Tella, 2001; Lobato et al., 2004; Gasparini et al., 2006; Bourgeon et al., 2010). In contrast, others show little or no effect of stress on immunity and thus conclude that the immune system is not a strong subject to trade-off (Råberg et al., 1998; Nilsson et al., 2007; Buehler et al., 2009). Van der Most et al. (2010) demonstrate a clear decrease in immune function when selecting for growth in poultry, but no negative effect on growth when selecting for immunity, which indicates a higher cost of growth than of immunocompetence.

Råberg et al. (1998) claimed that reduced immunocompetence at elevated CORT levels could actually protect the organism from autoimmunity or hyper-reactivity, and consequently be a defense mechanism rather than a trade-off. In accordance with this, Bourgeon et al. (2010) also found a clear reduction in adaptive and innate antibodies as CORT increased during different stages of fasting in mallards (*Anas platyrhynchos*). However, this relationship between stress and immunity was not observed during re-feeding. CORT levels returned to baseline levels within a day while natural antibodies recovered only slowly and adaptive antibodies remained at low levels. The authors consequently conclude that immune components not necessarily co-vary and that CORT not directly regulates immunocompetence, indicating a trade-off not entirely related to CORT but to energy status, and that the costs of different components differ.

Whether trade-offs in immunity occur during stress may be due to the evolutionary importance of maintaining high immunocompetence, or to the rate of an individual's exposure to stress before maturity (Martin et al., 2006; Martin, 2009). The benefit of down-regulating maintenance of the immune system is dependent on pathogenic challenges of the environment (Lochmiller & Deerenberg, 2000; Martin et al. 2005). Habitats at higher latitudes have in general a lower parasitic pressure which allows a reduced investment in immunity (Piersma, 1997; 2011; Martin et al., 2005). This might allow immunocompetence to be a greater subject to trade-off in stressful events, since it is less likely to reduce short-term survival (Martin et al., 2006).

1.6 Aim of study

In the present study I investigate the effect of altered stress of adult kittiwakes on offspring immunocompetence. Several studies on kittiwakes demonstrate that CORT is a good physiological indicator of food availability (Lanctot et al., 2003; Kitaysky et al., 2007; 2010), influence the timing of egg-laying (Goutte et al., 2010a; Goutte et al., 2011), and influence parental- and foraging behavior (Kitaysky et al., 1999). By increasing CORT release into the blood of parental birds during early chick-rearing, I stimulate a short-term period of increased food stress and investigate to what degree this higher allostatic load affects investment in reproductive effort. If parental investment indeed is compromised, I will investigate the effects on offspring immunocompetence and body condition. A decreased

immunocompetence assumingly indicates a reduced fitness in fledging kittiwakes that possibly could decrease long-term survival rates, but increase short term survival.

I predict that:

1) Offspring of parents with increased CORT release (CORT-implanted group) experience more food stress and hence increases CORT-levels, compared to control offspring (SHAM-implanted group)
2) Increased food stress in chicks will result in a trade-off between growth and immunocompetence, resulting in one of two scenarios:
    a. Body condition is maintained while immunocompetence is reduced.
    b. Body condition is reduced while immunocompetence is maintained.

2. Material and methods

2.1 Study area and model species

Field work was conducted between early July and mid-August in 2011 and 2012 in a colony of breeding kittiwakes in Kongsfjorden, Svalbard. The study colony is a medium sized colony of a few hundred kittiwake pairs situated on the island of Blomstrandhalvøya (78° 59'N, 12° 07'E). The colony experiences continuous daylight, little precipitation and with an average ambient temperature of +4.9°C during July (Meteorologisk institutt, 2013).

Kittiwakes are long-lived, pelagic, seabirds that nest on cliffs. They live throughout the northern hemisphere up to high-arctic regions and are the most abundant gull- species (Cullen, 1975; Strøm, 2006). Kittiwakes are monogamous and shares parental duties about equally (Strøm, 2006). The female usually lays one to three eggs per breeding attempt, although three eggs are less common. Often one or two semi-precocial chicks will fledge after 5-6 weeks of incubation (Strøm, 2006).

2.2 Field procedures

A thick nylon noose at the end of a fishing rod was used to catch adult kittiwakes by their neck, while chicks were accessed using a ladder. Blood from chicks and adults was taken from a brachial vein within 3 minutes of capture to measure baseline hormone levels unaffected by handling (Romero & Reed, 2005). Blood for hemolysis-hemagglutination assays were usually obtained within 3 minutes, and blood smears prepared within 8 minutes. A droplet of blood from all chicks and adults of unknown sex was placed in 96 % ethanol for later determination of sex by DNA analysis. Biometric measures were obtained during the first capture of each adult and chick by measuring body mass with a spring balance (±5 g), wing length with a ruler (±1 g), and skull (head + bill) and tarsus with a slide caliper (±0.1 mm). All measures were repeated when chicks were recaptured, while only body mass was measured in recaptured adults. Unbanded birds were banded with a metal ring from Stavanger Museum, Norway and a plastic ring to facilitate field observations from a distance.

In 2011, at an approximate chick age of 10 days, one parental kittiwake was implanted subcutaneously between the shoulders with a 25-mm silastic tube filled with crystalized CORT (n = 20). A control group received an empty implant (n = 20, SHAM). Treatments were randomly assigned. The implants were removed after about 72 hours. For the analysis of

baseline CORT, blood (<1 mL) was collected at each capture of implanted birds, and from both mates on a nest at approximately chick age 25 days. At this age, chicks were bled for a hemolysis-hemagglutination assay and two blood smears were made. Plasma for the hemolysis-hemagglutination assay was separated from red blood cells (RBC) within 5 minutes in the field by centrifuging the samples for 3 minutes at 9000 rpm. These samples were then immediately frozen in liquid nitrogen and later stored at -80 °C. Blood for hormone analysis were kept cold in the field, and then separated in the laboratory within 6-12 hours by centrifuging the samples in 3 minutes at 9000 rpm. Plasma was then stored at -80 °C. A drop of blood were placed on a glass slide and smeared to a thin, one celled layer. Blood smears were fixed in methanol for 1-2 minutes in field, before cells were stained by a Gimsa Stain within 2-3 weeks (The Schall Lab, 2011).

In 2012, blood samples (<0.5 mL) for CORT analysis were obtained from both mates of 27 pairs that were part of the 2011 experiments. Only a few pairs were either new to the 2011 nests or had changed partner since previous year. Three blood samples were taken throughout the season, the first during late incubation, and the second and third at approximate chick ages of 10 and 25 days, respectively. Additionally, chick were bled (<0.5 mL) at approximate ages of 10 and 25 days for CORT analysis, hemolysis-hemagglutination assay, and two blood smears were made (see above). Blood samples were handled as described above, except for hemolysis-hemagglutination samples which were kept on ice in field and stored at -20 C°.

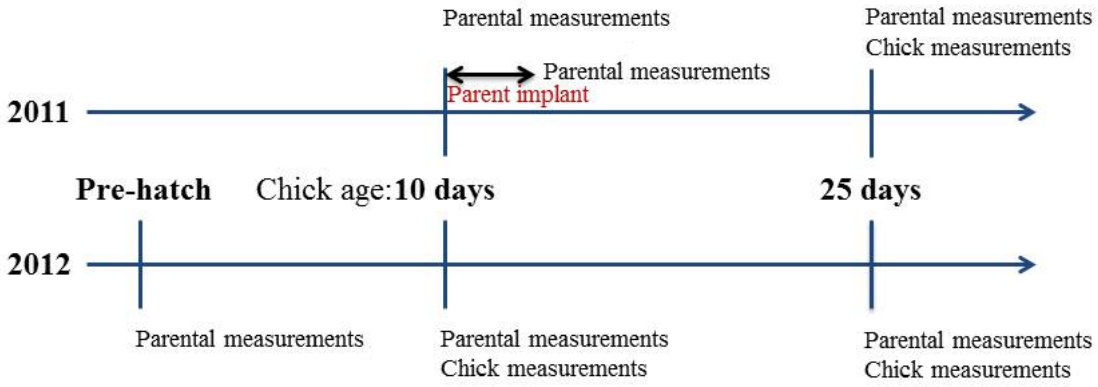

Figure 1. A timeline showing when measurements were taken during the field seasons of 2011 and 2012.

Storage temperature of plasma for hemolysis-hemagglutination assays for 2012 samples was not believed to cause any impact on the results, as they were analyzed within two months. Also, samples were not immediately frozen after separation in field in 2012, but as they were kept on ice for no more than six hours, I assume no effect on results, according to what was observed by Matson et al. (2005). NAbs are known to be less sensitive to short-term variations, such as stress, and should not have been affected by handling stress (Deerenberg et al., 1997). Blood smears were occasionally made later than 3 minutes (maximum 8 minutes). Previously published work (Buehler et al., 2009) and stress- tests conducted in 2012 (not presented in the present study) show no effect of handling time within this time-frame.

2.3 Hormone concentrations

Hormone concentrations were measured using a radioimmunoassay (RIA) method. Concentrations are detected by allowing radiolabeled (RL) and unlabeled antigens of the hormone compete for binding to a high affinity antibody (Kindt et al. 2007). RL antigen is mixed with high affinity antibodies to a point of antibody-antigen saturation. Plasma with unknown hormone concentration is then added as unlabeled antigen. Since the high affinity antibody does not distinguish between RL antigen and its unlabeled hormone antigen counterpart, the two antigens are equal competitors for antibody binding sites. The mixture is incubated for at least 24 hours to allow the reaction to take place. The larger concentration of hormone in plasma sample the more unlabeled antigen is bound to antibody. The hormone of interest is finally measured by determining the remaining bound RL antigen by detecting radiation with a gamma counter, after removing excess unbound RL antigen (Kindt et al., 2007).

Analysis of plasma concentrations of corticosterone were done according to Wingfield et al. (1992) at University of Alaska, Fairbanks (UAF), USA.

2.4 Measures of immunocompetence

Combining monitoring techniques (leukocyte profiles) and challenging techniques (Hemolysis-hemagglutination assays) should provide a representable measure of immunocompetence. Hemolysis-heagglutination assay is dependent on B-lymphocytes present in a H:L ratio, and B-lymphocytes are dependent on antigen marking by complement components, like the C3d, to recognizing antigens (Carrol & Prodeus, 1998). Thus, the

present measurements should interact, seen by a decrease in H:L ratio as lysis score increases. In the present study, extreme values are excluded to avoid influence by undesired factors.

2.4.1 Leukocyte profiles

Leukocyte profiles were measured by counting the number of erythrocytes occurring in one x1000 microscopic field and multiplying this number by the number of fields needed to count in total 100 lymphocytes (Dehnhard et al., 2011). The lymphocytes registered were monocytes (immature tissue macrophages), basophils, eosinophil, heterophils and leukocytes. The number of heterophils was then divided by the number of lymphocytes to estimate the ratio between important innate and adaptive lymphoid cells.

Before counting, blood smears were quickly scanned to find a representative area. Extreme numbers in cell counts were double checked. Scans were done along the short axis of the blood slide to get a representative selection of cells in the smear. Areas with multiple cell layers, cell clumping or cell destruction were avoided. Two blood slides were made for each sampling and thus counts were duplicated and then averaged. Several slides had to be excluded because of quality of the smear, thus only one slide were counted for some individuals.

2.4.2 Hemolysis-hemagglutination assay

By allowing serially-diluted plasma to react with RBCs from another species, in this case rabbit blood, NAbs binds to foreign antigens and cause agglutination, cell clumping, which allow effective recruitment and binding sites for complement molecules. Complement molecules cause lysis and destruction of foreign RBCs, thereby removing a possible threat to the individual. Both the level of agglutination and lysis are possible to quantify by scoring reactivity to which level of dilution and thus provide two parameters for innate immunity.

As NAbs are important for recruitment of cell-mediated immunity and an adaptive immune response, the hemolysis-hemagglutination assay both reflect an individual's ability to remove a threat instantly and to engage a stronger, specific adaptive response, which also provide a memory for later exposure. Unfortunately, agglutination scores were difficult to define unambiguously. The agglutination scores were therefor excluded, while lysis scores were kept.

The assay was carried out as originally described by Matson et al. (2005). All samples were analyzed at NTNU. Rabbit whole blood, preferred to test avian NAbs because of strong reactivity (Matson et al., 2005), was obtained from Harlan Laboratories in 50% Alsever's as anticoagulant. RBCs were washed four times with Dulbecos phosphate buffered saline (PBS) (spun at 2-5000 rpm). Hematocrit values were checked by the average length of RBC columns in two centrifuged capillaries filled with whole blood. Finally, RBCs were diluted to a concentration of 1% in PBS.

Round-bottom 96-well assay plates were used in tests by adding 25 µl of sampled plasma to the two first column of one row, in total 50 µl plasma. In row A and H, controls of pooled chicken plasma were added in the same way. 25 µl of PBS were added from column 12 to 2 in each row to avoid contamination. Content of wells in column 2 were mixed by use of a pipette and 25 µl mix were transferred to the next column (column 3), mixed and repeated until column 11, where 25 µl of final mix were thrown away. Finally, 25 µl of 1% RBC suspension were added by use of a multi-channel pipette to all wells from column 12 to 1. The plate were rotated horizontally in 30 seconds, before covered with parafilm and incubated in 37°C degree water for 90 min, to resemble a reaction at body temperature. After incubation, plates were cooled for 20 minutes at a 45° angle before scan for agglutination. Then put flat for another 70 minutes and scanned yet again for lysis.

From the image scans, agglutination and lysis were scored as negative $\log_2$ of the last column expressing positive behavior. Scores were given as whole or half points (Figure 2). Agglutination were decided from 20 minutes scans and express activity of NAb, while 90 minute (70 minutes after) scans expressed level of lysis and interaction between NAb and complement.

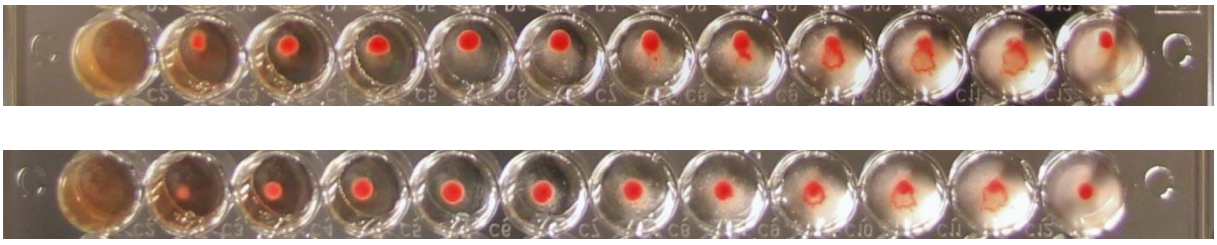

Figure 2. Row C, plate 9, 20 min scan shows an agglutination score of 6.5 (top picture). Row C, plate 9, 90 min scan shows a lysis score of 2.5 (bottom picture).

2.5 Molecular sexing

Blood for molecular sexing was analyzed at the Department of Biology at NTNU. Avian erythrocytes contain DNA and can be used for sexing by determine if certain DNA sequences are present in a sample. Analyzing presence of two sequences belonging to one of each chromosome would reveal one for the Z chromosomes in males, and two for the Z and W chromosomes in females. The DNA sequences are located by primers that bind and copy the sequences several times by a Polymerase Chain Reaction (PCR). Adding primer and the four nucleotides of DNA to a sample, the desired sequences are reproduced during 35 heat-cycles. The different lengths of the sequences are then identified by gel electrophoresis. The negative charged DNA-sequences are loaded into wells of a gel and pulled towards the positive charged side of the gel when adding electricity. The length of the sequences decides how fast they move. Gender is determined by a single (male) or double (female) lines when gel is exposed to UV light.

Analyses were done according to Griffiths et al. (1998). DNA were extracted from the sample in vitro by taking a small amount of blood (2-4 µL) and adding it to a 200 µL 5% Chelex mixture. Mixture were heated at 56 °C for ca 20 minutes, vortexed, and heated again at 96 °C for exactly 8 minutes. After centrifuging (12000 rpm, 3 min) 20 µL of supernatant were kept for DNA analysis. A stock mix containing primers and nucleotides were made by adding Taq (0,5 µL), H_2O^{∞} (1,95 µL), Mix (0,40 µL), MgCl (0,60 µL), 10X (1 µL), primer 2718 (100µM, 1µL), primer 2550 (100 µM, 1 µL) and Q (2 µL). 2 µL DNA supernatant and 8 µL stock mix were added to a well of a PCR plate and run in a premade program for Kittiwakes, where heated for 3 minutes at 94.0 °C before run in 35 heat cycles: 94.0 °C 0:30, 46.0 °C 0:45, 70.0 °C 0:45, and then 70 °C 10 min before cooling down to 4 °C. A 1 % gel were made of 1,2 mL 50x TAE buffer, 59 mL H_2O^{∞}, 0,6 g agarose and 6 µL gel stain. A running buffer (686 mL $H_2O^{\infty}$and 14 mL 50xTAE buffer) was added to the gel-chamber and the sample was loaded into a well of the gel. The gel was run for 45 min at 75 volt and DNA strains identified by UV light.

2.6 Statistical analysis

All computations were performed with R statistical software (R Core Team, 2011) and all graphs made with SigmaPlot 12.3 (Systat System Inc., 2011). Data was tested for normal distribution by plotting sample residuals against theoretical residuals and with a Shapiro-Wilk test of normality. The level of significance tests was set at $P \leq 0.05$ for all tests. Relationships

between two continuous variables were analyzed with Pearson correlation tests. A Welch's t-test was used to test for differences between groups for continuous variables.

Body condition index (BCI) was calculated separately for each gender of adult birds since they differ in body size, but not for nestlings, since gender is not believed to affect phenotype before a chick age of 25 days (C. Bech, unpublished data). Lengths of wing and tarsus correlated poorly with body mass therefore only residuals from plotting skull length against body mass were used as BCI in adult birds. A size index for chicks was calculated with a principle component analysis (PCA), using age and skull length as predictors. The residuals of PC1 against body mass were used as BCI.

A candidate set of models was created to test contrasting hypotheses for effects on different components of chick fitness. Several linear models were tested to explain variations in the response parameters lysis, H:L ratio, chick CORT and chick BCI. Only birds that still had chicks at the latest sampling point in each season were included in the models.

Candidate models for each response parameter:

Model 1: chick response parameter ~1 (Null model)
Model 2: chick response parameter ~Parental CORT (mean values of both mates)
Model 3: chick response parameter ~Parental BCI (mean values of both mates)
Model 4: chick response parameter ~Chick sex
Model 5: chick response parameter ~Sibling
Model 6: chick response parameter ~Chick age
Model 7: chick response parameter ~Group (only for 2011)

Candidate models were selected by their hypothesized ability to affect nestling fitness. The best model set of models was selected by running through an Akaike Information Criterion (AIC) analyses corrected for small sample sizes (AICc). The model with the lowest ΔAICc value was regarded as the best model, but any model with a $\Delta AICc < 2$ to the best model was regarded as indistinguishable (Burnham & Anderson, 2002).

If differences in chick fitness, for those of 2011, were affected by treatments, model 2 or 7 should be selected. If influenced by parents, but not treatments, model 3 should be selected. If variations in chick fitness were caused by other factors, such as sexual size dimorphism (SSD), sibling competition or differences in age, model 4-6 should be selected, respectably. If variations could not be sufficiently explained by the other candidate models, a null model should be selected (model 1).

When selecting models for all chicks (2011 and 2012), the group model was excluded, as it was not fit in this context to explain whether or not treatments influenced on selection. Parental CORT (2012, model 2) were therefor left as the only model indicating if treatments still influenced selection. All other models were kept.

Hormonal and immunological parameters were not affected by handling time, and thus no correction was done.

2.7 Permissions

All fieldwork was approved by the National Committee for Animal Research in Norway (Forsøksutvalget; ref. 2011/65070) and the Governor of Svalbard (Sysselmannen; ref. 2011/00488-25; 2010/00382-10).

3. Results

3.1 Physiological effects of CORT-manipulations

Endocrine manipulations of parental birds reduced circulating CORT significantly 3-day post-implantation and later (Figure 3). The initial levels of CORT during early chick rearing did not significantly differ between the three groups (2011 CORT, 2011 SHAM and 2012). Individuals exposed to exogenous CORT experienced a significant decrease in hormone levels towards late chick age ($p = 0.032$, Table 1), while changes in CORT were non-significant in other control individuals. CORT levels were also significantly lower in CORT-treated individuals, compared to individuals from SHAM and 2012 groups at late chick age (CORT-SHAM, $p = 0.004$; CORT-2012, $p = 0.001$, Table 2). Hence, implantations of exogenous CORT had an effect on parental kittiwakes. However, when considering mean hormonal values of both implanted and non- implanted mates of treatment groups (2011 CORT, 2011 SHAM), differences were non-significantly lower at late chick age ($p = 0.075$) in CORT-treated compared to SHAM-treated pairs.

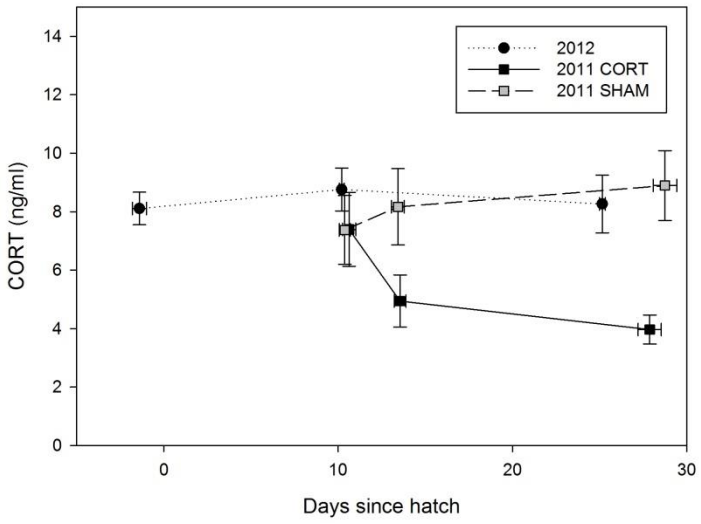

Figure 3. Parental CORT values as a function of days since hatch, with CORT-treated (solid line, filled square), SHAM-treated (dotted line, open squares), and non-treated 2012 (dotted line, filled circle) individuals. The values presented are means with ± SE.

Treatments did not seem to affect parental BCI, as none of the parental groups changed significantly between early and late chick age (CORT, $p = 0.606$; SHAM, $p = 0.945$; 2012, $p = 0.319$, Table 3). However, SHAM implanted individuals had a lower BCI than that of other groups, just barely non-significantly different from CORT-implanted individuals at early chick rearing ($p = 0.057$). Pairs (implant + mate) between the two implant treatments were also non-significantly different in their mean BCI at late chick age ($p = 0.105$).

Table 1. Results from paired t-tests investigating change in parental mean CORT between initial baselines at time of pre-implantation (chick age ~ 10 days) and approximately 70 hours (chick age ~ 13 days) and two weeks later (chick age ~ 25 days). Presented here are t-values (t), sample sizes (n), p-value (*P*) and changes in CORT levels (ΔCORT). Significant p-values are indicated in bold.

Group		**t**	**n**	**ΔCORT**	***P***
CORT	10/13 days	1.148	7	-2.024	0.289
	10/25 days	2.790	6	-3.889	**0.032**
SHAM	10/13 days	-1.127	7	1.02	0.297
	10/25 days	-1.909	7	2.332	0.098
2012	10/25 days	0.607	35	-0.602	0.548

Table 2. Results from Welch's t-tests of Figure 3, investigating differences in CORT between the parental groups at time of pre-implantation (chick age ~ 10 days) and approximately 70 hours (chick age ~ 13 days) and two weeks later (chick age ~ 25 days). Presented here are mean CORT values (CORT, SHAM, 2012), sample sizes (n), and p-values (*P*). Significant values are indicated in bold.

	CORT	**n**	**SHAM**	**n**	**2012**	**n**	***P***
Day 10	7.395	8	7.377	8			0.992
Day 10			7.377	8	8.620	35	0.394
Day 10	7.395	8			8.620	35	0.429
Day 13	4.944	9	8.402	8			**0.033**
Day 25	3.969	8	8.897	8			**0.004**
Day 25			8.897	8	7.949	28	0.546
Day 25	3.969	8			7.949	28	**0.001**

Table 3. Results from paired t-tests investigating change in parental mean BCI between pre-implantation (chick age ~ 10 days) and approximately two weeks later (chick age ~ 25 days). Presented here are t-values (t), sample sizes (n), p-value (*P*) and changes in BCI levels (ΔBCI).

Group		**t**	**n**	**Δ BCI**	***P***
CORT	10/25 days	-0.544	6	4.335	0.606
SHAM	10/25 days	0.072	7	-0.511	0.945
2012	10/25 days	1.029	16	-3.542	0.319

Table 4. Results from Welch's t-tests, investigating differences in BCI between the parental groups at time of pre-implantation (chick age ~ 10 days) and approximately 70 hours (chick age ~ 13 days) and two weeks later (chick age ~ 25 days). Presented here are mean BCI values (CORT, SHAM, 2012), sample sizes (n), and p-values (*P*).

	CORT	**n**	**SHAM**	**n**	**2012**	**n**	***P***
Day 10	-9.882	9	-23.719	8			0.105
Day 10			-23.719	8	-10.588	20	0.057
Day 10	-9.882	9			-10.588	20	0.919
Day 25	-5.677	9	-21.524	7			0.151
Day 25			-21.524	7	-14.630	17	0.422
Day 25	-5.677	9			-14.630	17	0.233

3.2 Differences in chick fitness

Chicks from CORT manipulated nests showed a tendency towards better fitness (i.e. higher lysis score, lower H:L ratio, lower baseline CORT, higher BCI), compared to nestlings from SHAM- and 2012-nests (Figure 4). However, only BCI was significantly different between CORT and SHAM nestlings of 2011, with a higher BCI in chicks of CORT-implanted parents (p = 0.027, Table 5). Chicks of CORT-implanted individuals also had both significantly higher immunocompetence (low H:L ratio, high lysis score) and lower circulating CORT than nestlings of 2012 (H:L, p = 0.002. Lysis score, p = <0.001. CORT, p = 0.037, Table 5).

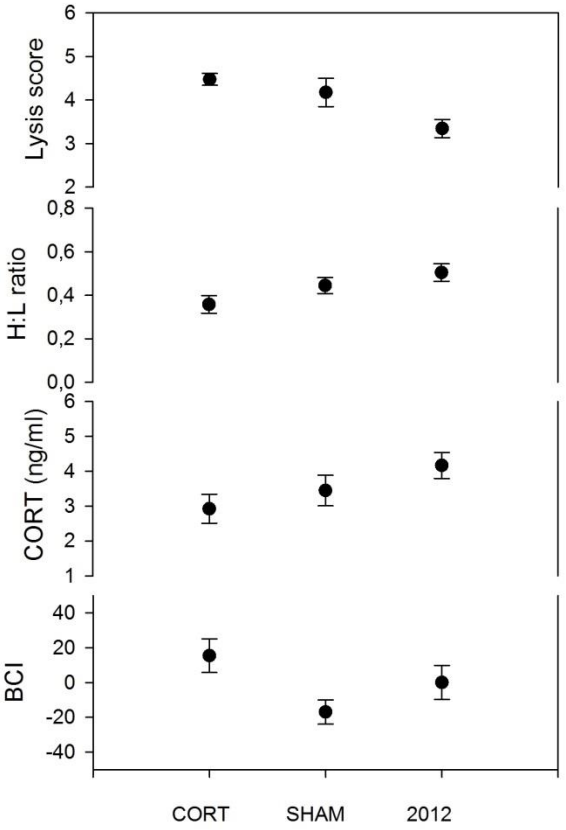

Figure 4. Immune parameters, CORT level and BCI in nestlings of CORT- and SHAM-implanted parents, together with non-experimental nests of 2012. The values presented are means ± SE.

Table 5. Results of Welch t-tests investigating differences in response variables between chicks of CORT- and SHAM-implanted parents, and between nestlings from CORT-implanted and non-experimental parents of 2012, based on values in Figure 4. Shown are means, sample sizes (n), and p-value (*P*). Significant values values are shown in bold.

Response variable	**CORT**	**n**	**SHAM**	**n**	***P***	**CORT**	**n**	**2012**	**n**	***P***
H:L ratio	0.358	11	0.445	10	0.128	0.358	11	0.531	19	**0.002**
Lysis score	4.475	11	4.175	10	0.411	4.475	11	3.348	22	**<0.001**
CORT	2.921	10	3.447	10	0.397	2.921	10	2.931	21	**0.037**
BCI	13.808	11	-16.956	10	**0.027**	13.808	11	0.001	22	0.346

Time of day did not correlate with any of the response parameters, except in lysis scores of 25 day old chicks (Figure 4). However, lysis scores in CORT-treated nests alone, where sampling occurred throughout the day, showed no such tendency. In addition, samples in SHAM-treated nests in 2012, both of which show comparably low lysis scores, were mostly collected during early hours and may cause a bias in the overall trend. The overall relationship between time of day and lysis scores was thus mostly driven by two chicks from SHAM-treated nests that were sampled during late hours. Finally, only few records of the exact time of day were made during 2012 sampling. However, when estimating sampling time based on the few records available, combined with the order of samples taken during that day (sampling time therefor ±h-2 hours), I did not find a significant relationship between daytime and immunity.

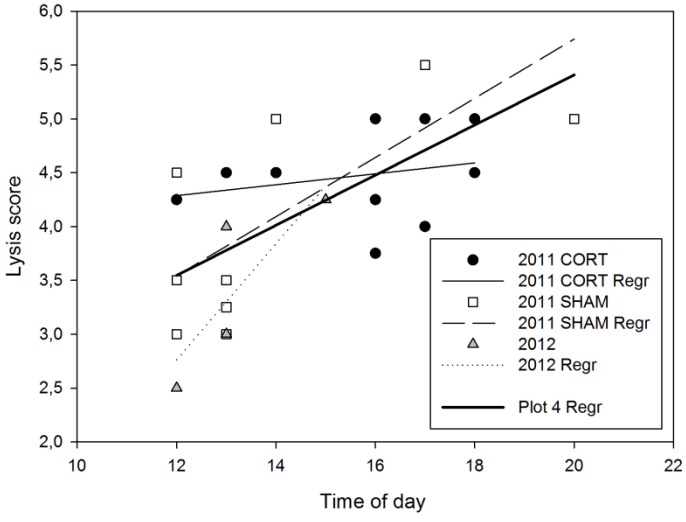

Figure 5. Lysis score measured in plasma from nestlings of **(1)** CORT-treated (closed circles), **(2)** SHAM-treated (open circles) and **(3)** 2012 non-treated (open triangles) nests presented against time of day. Each group are represented by thin solid (CORT), medium dashed (SHAM) and dotted (2012) regression lines. All groups represented with an thick, solid regression line

H:L ratios and lysis scores correlated negatively ($p<0.001$, corr = -0.0469; Figure 6). When excluding 10-day old chicks, this relationship became only a tendency ($p = 0.089$, corr = -0.279). Sample size could cause a non-significance (type II error) when only considering nestlings of approximatly same age.

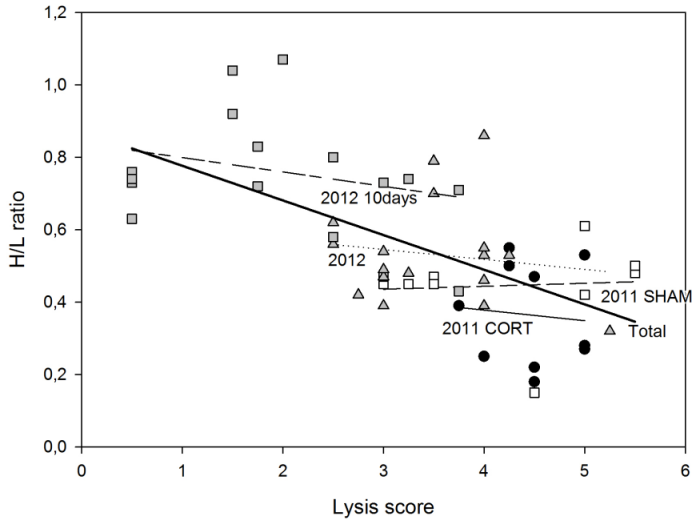

Figure 6. Relationship between H:L ratios and lysis score in nestlings of **(1)** CORT-treated nests (25 days old, filled circles, solid regression line), **(2)** SHAM- treated nests (25 days old, open squares, medium dashed regression line), **(3)** 2012 nests (10 days old, gray squares, short and medium dashed regression line), **(4)** 2012 nests (25 days old, gray triangles, dotted regression line). Solid, thick regression line shown for all groups. Overall regression line for 25 day old chicks not shown.

3.3 Effects of CORT-manipulations on chick fitness

Multiple chick fitness parameters were affected by parental stress, based on model selection results (Table 6). Firstly, parental CORT treatment during early chick-rearing affected chick body condition and chick baseline CORT levels (Table 6a). Secondly, parental CORT levels during late chick-rearing affected chick lysis scores and chick CORT levels (Table 6a). The H:L ratio were not affected by treatments. When combining data for 2011 and 2012, parental CORT levels during late chick-rearing affected lysis scores, chick BCI and chick CORT levels (Table 6b). Parental BCI, in contrast, did not explain variation in chick fitness (see Appendix for complete results).

There were skewed distributions of B-chicks, male-female chicks, and chick age. Among SHAM chick, there were more B-chicks (n = 3), compared to CORT chicks (n = 1; see

Appendix, Table A). Also, nestlings in 2012 were about 1.4 days younger when sampled compared to sampling in 2011. Both these distributions between the groups affected chick CORT (Table 6a&b), and H:L ratios (Table 6b). More female chicks in SHAM nests compared to those with CORT-treated individuals also affected chick CORT, lysis score (Table 6a) and H:L ratio (Table 6a&b).

Table 6. Results of an AICc test for model selection investigating parental effects on chick parameters. Models were selected from a set of candidate models (see Appendix), with all models with ΔAICc < 2 regarded as indistinguishable. All chicks were about 25 days old (24-29 days). CORT nestlings, n = 11. SHAM nestlings, n = 10. 2012 nestlings, n = 23.

Response variable	Candidate model	ΔAICc	AICc weights (%)
	2011 only		
H:L ratio	Chick sex	0.000	75.6
Lysis score	Chick sex	0.000	33.0
	Null model	0.699	23.2
	Parental baseline CORT	1.483	15.7
Chick baseline CORT	Null model	0.000	28.2
	Parental baseline CORT	0.971	17.3
	Sibling	1.387	14.1
	Chick age	1.681	12.2
	Chick sex	1.901	11.0
	Parental CORT treatment	1.974	10.5
Chick BCI	Parental CORT treatment	0.000	65.2
	2011 & 2012		
H:L ratio	Chick sex	0.000	40.7
	Chick age	1.081	23.7
	Null model	1.462	19.6
Lysis score	Parental baseline CORT	0.000	64.4
Chick baseline CORT	Parental baseline CORT	0.000	97.8
Chick BCI	Parental baseline CORT	0.000	100.0

Model selection shows an effect of chick gender on immunocompetence. Both measured parameters show better immunocompetence in males when including all chicks (H:L ratio, p = 0.0158; Lysis score, p = 0.057, Figure 7a&b). By excluding chicks of CORT-treated

individuals the tendency remained the same, however differences were non-significant in both parameters (Figure 7c&d).

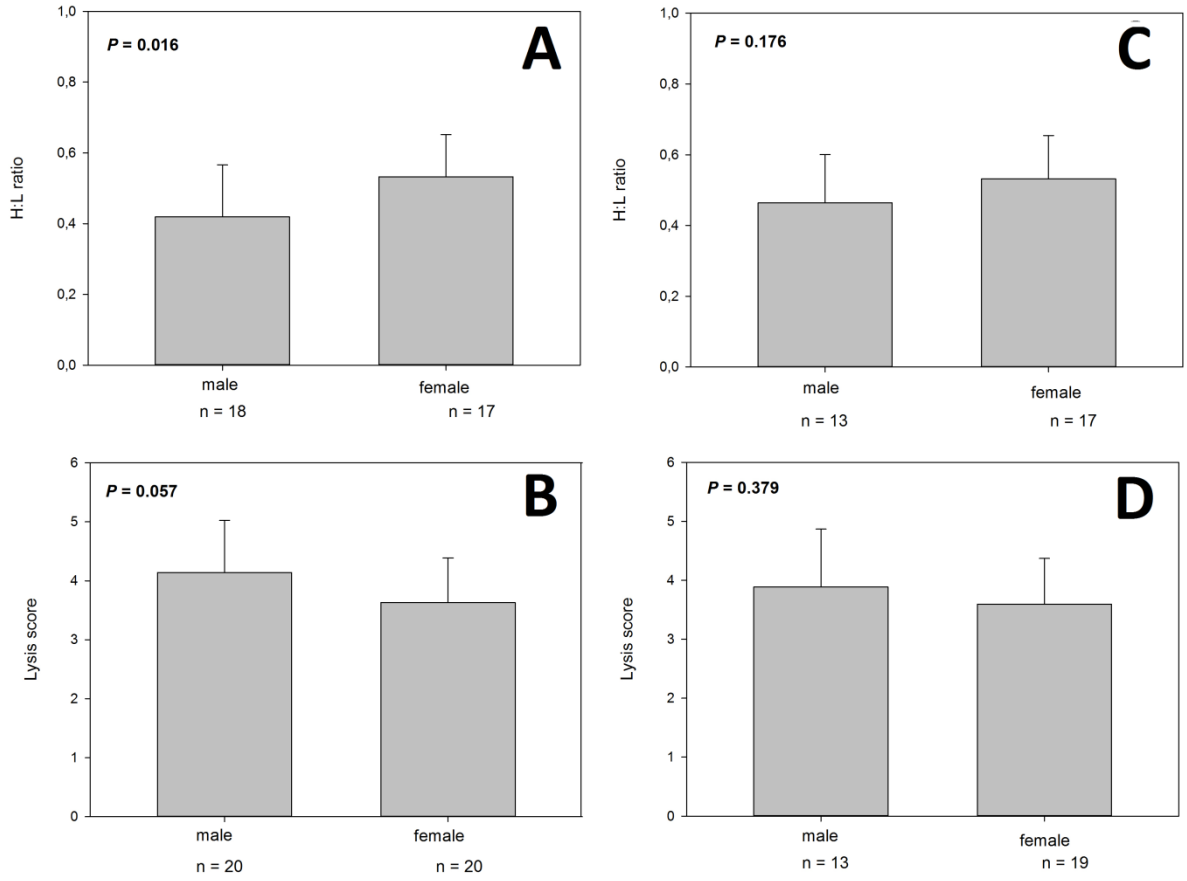

Figure 7. Parameters of immunocompetence in male and female kittiwake chicks of approximatly 25 days of age. (A) H:L rartio off all measured chicks. (B) Lysis scores of all measured chicks. (C) H:L ratio of chicks of SHAM and 2012-groups. (D) Lysis scores of chicks in SHAM and 2012-groups. P-values and standard deviation are shown.

4. Discussion

In the present study I found an unexpected decrease in baseline CORT in stress-manipulated individuals of parental kittiwakes, with CORT values being 55% lower than that of SHAM-treated individuals at the latest point of sampling. This was accompanied by a greater overall fitness in the offspring of CORT- manipulated adults.

4.1 Effect of CORT treatments

CORT manipulations have been used in several stress-related studies of Kittiwakes. Most of these studies have been done with the intention of increasing the stress levels (Kitaysky et al., 2001; Kitaysky et al., 2003; Angelier et al., 2007; 2009; Goutte et al., 2010b), but also reducing them (Goutte et al., 2011; Shultz & Kitaysky, unpublished). With the use of implants as in the present study, CORT usually peak within the first 48 hours, before returning to previous baseline within a few days after implantation (Kitaysky et al., 2003; Angelier et al., 2009; Goutte et al., 2010b). As the length of exposure, from a few days to weeks, and amount of exogenous CORT used varies greatly between the above studies, the physiological reaction to the treatments may also differ. This has led to various interpretations of what mechanisms could come at play when exposing birds to exogenous CORT. The manipulations used in the present study were assumed to be within the physical range of a natural stress response, representing a short-term event of increased food stress. However, the same method was applied by Goutte et al. (2011) in order to decrease stress.

Goutte et al. (2011) argued for a negative feedback mechanism that reduces the activity of the HPA axis. By decreased expression of corticotropin-releasing hormone (CRH) genes in the hypothalamus, CRH receptors are further reduced in the pituitary, and stimulation of adrenocorticotropic hormone (ACTH) secretion is thereby inhibited, resulting in low endogenous CORT (Dallman et al., 1987; Aguilera et al., 2001; Fieldman & Weidenfield, 2002; Vanderborne et al., 2005). Inhibition by use of exogenous CORT has also been induced in other studies by use of silastic tubes (Romero et al., 2005), and with pellets (Müller et al., 2009). These last authors recorded a down-regulation of the HPA axis in kestrels (*Falco tinniculus*) which resulted in an absence of an acute stress response during the sequent eight days after implantation. A reduced expression of the HPA axis was also discussed in Goutte et

al. (2010b), where long-term survival in kittiwakes were lower in CORT manipulated individuals compared to controls. In the study by Goutte et al. (2010b) silastic tubes were used with the intention of exposing individuals for exogenous CORT for a few days, which in reality may have been for weeks, as they were removed first two years later, and thus have caused chronic responses. On the other hand, Kitaysky et al., (2003) argued for increased stress during long-term exposure (weeks) of exogenous CORT, by higher turnover rate of circulating CORT. This can mask the actual CORT exposure to target tissues as CORT return to baseline. My study provides positive effects on response variables, as I observed a tendency towards higher nestling immunocompetence and BCI in response to low parental CORT (discussed more in detail below). This is in accordance with what was observed by Goutte et al. (2011).

Multiple physiological mechanisms could interact with CORT to modulate the stress response. CORT circulate in the blood in equilibrium with CORT binding globulins (CBG) and in a free state. According to the Free Hormone Hypothesis, CBG-bound CORT is believed to function as an immediate reservoir of CORT, while free CORT is the biological active fraction which is readily absorbed by target tissues (Malisch & Breuner, 2010). Whether or not measuring only total CORT is sufficient in relation to the Free Hormone Hypothesis is a hot topic in the present discussion of measuring endocrine stress, since free and CBG-bound can mask the metabolic turnover of CORT if the equilibrium is regulated in a stress response. This has been seen in House sparrows (*Passer domesticus*; Breuner & Orchinik, 2001; Romero et al., 2006), but not in pacific kittiwakes (Shultz & Kitaysky, 2008). In the present study, total CORT will be regarded as representative for measuring stress.

4.2 Measuring Immunocompetence

Measuring one single component to assess immunocompetence may be too simplistic, as the immune system is very complex, and misinterpretations might occur if the results are not interpreted with caution (Apinius, 1998b; Norris & Evans, 2000). In the present study I have combined indirect and direct measurements of immunity by measuring both the H:L ratio, and NAb- and complement caused lysis, in order to assess immunocompetence. Hetrophil and lymphocyte counts has been demonstrated to reflect the ontogeny of the immune system well in Red-tailed tropicbirds (*Phaeton rubricauda westralis*; Dehnhard et al., 2011), and recruitment in nestlings of Pied flycatchers (*Ficedula hypoleuca*; Lobato et al., 2005), while

lysis score has proven a good indicator for NAb and complement levels in various avian species (Kohler et al., 2003; Matson et al., 2005). The two immune parameters were expected to interact, since complement mediates activation of B-lymphocytes, and NAbs (IgM) are produced by B-lymphocytes. Comparing nestlings of approx. 10 and 25 days of age resulted in a significant correlation between H:L ratio and lysis score, paralleling growth and development of the lymphoid organs (Apinius, 1998b, Møller et al., 2003). The low level of lysis at early chick age also indicates little influence of maternal transferred IgY antibodies, and so it reflects the chicks' own immunity, in line with what has been previously shown (Matson et al., 2005). Hence, it is safe to assume that the measured components are linked and together can be used to asses immunocompetence.

Misinterpretations could occur if environmental factors such as daytime variation affect immunocompetence. I observed a tendency for higher lysis scores towards late daytime (Figure 5). Variation in immunity to time of day has also been observed in T-cell-mediated immunity in nestlings of the Eurasian kestrel (*Falco tinnunclus*; Padilla, 2006). In the present study the midnight sun does not provide any strong photoperiodic cue for activity or immune regulation, in contrary to Padilla's study (Padilla, 2006). Estimated time of day to lysis scores for chicks from 2012 revealed no tendency towards variation between daytime and immunity, as well as for the H:L ratios. Thus, my findings do not support any effect of daytime on immunocompetence.

4.3 Parental influence on chick fitness

As the dominant paradigm assume that elevated stress reduce parental investment (Kitaysky et al., 2007), one should expect decreased endocrine stress to have the opposite effect. The two breeding seasons during which the present experiments were carried out, provided normal foraging conditions and no extreme weather events. The experimental lowering of baseline CORT did not influence BCI in parental birds. Instead, offspring were found to have higher fitness by having higher immunocompetence, higher BCI and lower stress than the offspring of SHAM and non-treated (2012) parents (Figure 4). Variation in fitness was also found to be linked to both parental level of CORT and relation to experimental treatment groups, which indicate an enhanced parental effort in manipulated birds by e.g. increased nutritional availability for offspring. This is in accordance with a fixed investment hypothesis (Welcker et al., 2010), where decreased stress within normal foraging conditions would increase energy

available for parental reproductive effort, as parental self-maintenance already should be sufficient.

Weight of immune related organs is positively correlated to the immune responses (Møller et al., 2003). The developing rate and weight of these organs can be influenced by several factors, such as nutrient availability, SSD, age, genetic components or sibling competition. An enhanced parental effort is assumed to increase nutrient availability for offspring, either in mass or quality of food, and further increase growth rate and development of chicks, affecting both immunocompetence and BCI.

High predation during the breeding season of 2011 led to a low sample size of chicks of implanted individuals. In the statistical model selection the presence of several null models and low AICc weights indicate that the factors was influenced by the relative low sample size of 2011 (in total, n=21) that may mask the effects of hormone manipulations. Models selected for CORT levels in chicks would be the best example. Here, 6 out of 7 models were selected for having explanatory value for the hormonal value in chicks, leaving their actual influence on chick CORT highly inconclusive. However, when including chicks of 2012, parental baseline CORT was selected as the single best explanatory model for chick CORT. Hence, chick CORT is regarded as being influenced by parental CORT.

4.4 Impacts of age, sexual dimorphism and sibling competition

Rates of sex, age and sibling competition between different groups had somewhat skewed distributions that could inflict on the results. The majority of study nests hatched two chicks, and thus most nestlings were subject to sibling competition at some point. Between the treatment groups of 2011, SHAM nests contained more second hatched siblings than nests of CORT-treated parents at the latest time of sampling. This distribution could have influenced fitness, as younger siblings are known to fledge in poorer condition than older siblings or single hatched chicks of kittiwakes (Coulson & Porter, 1985). Sibling competition had an impact on chick CORT parameters of 2011, among many other factors for this particular parameter, but did not seem to affect any other measured components of fitness. Hence, sibling competition is assumed to have little influence on the results.

Sexual size dimorphism has been shown to influence immunocompetence in several avian species, where males tend to have lower immunocompetence than females (Fargallo et al.,

2002; Müller et al., 2003; Tschirren et al., 2003). In species where male size is selected for, as in kittiwakes, males might be forced to perform trade-off between increased size and other costly traits, such as immunocompetence (Immunocompetence handicap hypothesis; Folstad & Karter, 1992). Immunosuppressive effects of the sexual related hormone testosterone is a much applied explanation for the difference in immunity (Koutsos & Klasing, 2008; but see Hasselquist et al., 1999). However, no sexual difference in chick immunity was observed in a previous study of kittiwakes (Noreen, 2007). In the present study immunocompetence were strongly affected by sexual dimorphism when including chicks of CORT-treated parents (Figure 7a), with a higher immunocompetence in male chicks. SHAM-treated nests contained more female chicks than CORT-treated nests. These differences changed to being non-significant when excluding CORT chicks. Hence effects of the parental manipulations might have exaggerated sexual differences in immunocompetence, but also the differences in distribution of genders could have masked effects of treatments on immunocompetence. However, H:L ratios still remained higher for males (figure 7), which is not in line with what has been observed in other studies of T-cellular immunity in chicks.

The lower age of sampled chicks from 2012 had some influence on the H:L ratios. Hence age contributed to differences in immunocompetence between chicks of 2012 and 2011, which might indicate less developed lymphoid organs. However, lysis scores were not affected, which might be caused by less sensitivity or other developing rates in relation to age. For example are complement molecules not solely dependent on lymphoid tissues, but are also developed in spleen and other organs (Kindt et al., 2007). Antibodies have also been reported to decrease after the first 20 days of age of inbreed lines of chicken (Rees & Nordskog, 1981), which can counteract differences in age.

4.5 Immunocompetence in allostasis

Nestlings have consistently shown to be food limited in many experimental studies (Martin, 1987). When conditions were improved in the present study, only some components of immunocompetence were affected. H:L ratios were unaffected and lysis scores were influenced by several other factors as well as for variation in parental stress. In contrast, BCI was clearly affected by treatments, which indicate a higher allocation of resources towards increased BCI than immunocompetence in kittiwake chicks. Alonso-alvarez and Tella (2001) suggested a threshold to where improvement of immunocompetence could occur in the yellow-legged gull (*Larus cachinnans*). As the kittiwakes live in areas characterized by

having less parasitic pressure (Piersma, 1997) their environment may not strongly select for a higher than normal immunocompetence, but rather select for an enhanced somatic growth when conditions are improved. Older nestlings face a period of less parental feeding paralleled with gain of flight capacity (Coulson & Porter, 1985). Higher body reserves might increase short-term survival probability under these changing life conditions, as fledglings are getting increasingly dependent on their self-sufficiency. A higher immunocompetence could possibly limit energy flexibility during this critical stage.

A study on American kestrels (*Falco sparvericus*) found an increase in immune function with a small, non-chronic, increase of nestling CORT, compared to the control group (Butler et al., 2009). This finding does not support a threshold of increased development in immunocompetence. Also, in several nutritional studies of avian species, immunocompetence has been shown to respond positively to protein richness in the diet, as seen in barn swallows (*Hirundo rustica*) and northern bobwhites (*Colinus virginianus*), where nestlings have increased immunocompetence by cell mediated immunity, but no increase in body mass, when fed with a high-protein diet (Lochmiller et al., 1993; Saino et al., 1997). However, trade-offs in immunity is likely to be highly dependent on the life-history of the studied species.

When food stress increases, either by reduced food availability or by altered nutrient status, studies have found a maintained BCI at the expense of decreased immunity (González et al., 1999; Alonso-alvarez & Tella, 2001). During food deprivation, energy might be allocated away from immunocompetence, to maintain other body functions that would increase survival during this critical event. However, down-regulation of immunocompetence should be contextual depending on factors such as present life-stage, own body condition and the nature of the energetically challenge. For example is immunocompetence down-regulated during long-distance migration of many migratory species, but long experiments with continuous flights in a wind tunnel did not considerable suppress immunity in the Red knot (*Calidris canutus*), which might indicate anticipatory regulation in allostasis (Hasselquist et al., 2007).

The cost of maintaining immunocompetence is difficult to assess and the benefit of suppressing immunity is uncertain. The present study indicates that body condition is of high importance for kittiwake chicks, which has also been seen in earlier works on survival rates of fledging kittiwakes (Coulso & Porter, 1985; Cam et al., 2003). An increase in immunocompetence beyond normal levels could be less prioritized in this life-stage.

However, my results remain speculative concerning trade-offs in immunity in relation to increased food stress in kittiwakes.

4.6 Conclusion

The present study saw an enhanced parental effort when the activity of the HPA axis was inhibited by a short-term exposure of exogenous CORT in kittiwakes. This was expressed by a lower individual CORT, no change in BCI, and higher fitness in offspring. Hence, my results support a 'fixed investment hypothesis', in which parental birds will increase their reproductive effort, if food stress is decreased under normal foraging conditions. Increased parental investment in offspring resulted in a significantly higher chick BCI than in controls, while immunocompetence and levels of CORT were less affected by parental treatments. Hence, there was a greater allocation of energy towards higher body condition, rather than immunocompetence. This might imply a higher selection for body condition during development, when exposed to normal conditions. However, this study cannot predict trade-offs in immunity in the opposite event of increased food stress. This is the first study to present experimental data on immunocompetence in offspring of adult kittiwakes when the parental stress level is manipulated.

4.7 Future studies

At this point, no study on immunocompetence and survival has been performed on kittiwakes, but data on chick immunocompetence has been generated through several seasons on Svalbard, so it should be possible to compare to return rates and immunocompetence in future studies. Also, a small tendency towards lower H:L ratios were seen in male chicks in this study, but a larger amount of data is needed to see if there exist a possible difference between the genders.

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Appendix

Table A. Overview of sampled individuals of 2011 and 2012 at chick age of approx. 25 days. Parental birds of 2011 are divided into implanted birds and mates. Chicks are divided into A and B siblings in order of hatch. No third sibling survived until day 25.

Group	Adults		Chicks	
	Implanted	**Mate**	**A chick**	**B chick**
CORT	6 female	4 female	3 female	0 female
	4 male	6 male	7 male	1 male
SHAM	6 female	2 female	4 female	2 female
	2 male	6 male	3 male	1 male
2012	21 female		9 female	5 female
	21 male		8 male	1 male

Table B. Results of Welch t-test between response variables in nestlings of SHAM-treated pairs and pairs of 2012, based on Figure 4.

Response variable	**SHAM**	**2012**	***P***
HL ratio	0.445	0.531	0.087
Lysis score	4.175	3.348	**0.047**
CORT	3.447	2.931	0.397
BCI	-16.956	0.001	0.167

Table C. Results of Welch t-test between response variables in nestlings of pairs from 2011 and 2012, based on Figure 4.

Response variable	**2011**	**2012**	***P***
HL ratio	0.405	0.531	**0.000**
Lysis score	4.325	3.348	**0.001**
CORT	3.184	2.931	0.606
BCI	-1.575	0.001	0.501

AICc tests with chicks of 2011

Table D. Results of an AICc test for model selection investigating parental effects on chick H:L ratio. Models were selected from a set of candidate models, with all models with ΔAICc < 2 regarded as indistinguishable. All chicks were about 25 days old (24-29 days). CORT nestlings, n = 11. SHAM nestlings, n = 10.

Response variable	Candidate model	K	AICc	Delta AICc	AICcWt
H:L ratio	Chick sex	3	-25.412	0.000	0.756
	Parental CORT treatment	3	-20.660	4.752	0.070
	Null model	2	-20.355	5.057	0.060
	Chick age	3	-20.268	5.145	0.058
	Parental baseline CORT	3	-18.464	6.948	0.023
	Parental BCI	3	-17.750	7.662	0.016
	Sibling	3	-17.734	7.679	0.016

Table E. Results of an AICc test for model selection investigating parental effects on chick lysis scores. Models were selected from a set of candidate models, with all models with ΔAICc < 2 regarded as indistinguishable. All chicks were about 25 days old (24-29 days). CORT nestlings, n = 11. SHAM nestlings, n = 10.

Response variable	Candidate model	K	AICc	Delta AICc	AICcWt
Lysis score	Chick sex	3	48.604	0.000	0.330
	Null model	2	49.303	0.699	0.232
	Parental baseline CORT	3	50.0864	1.483	0.157
	Parental CORT treatment	3	51.455	2.851	0.079
	Chick age	3	51.562	2.958	0.075
	Sibling	3	51.849	3.245	0.065
	Parental BCI	3	51.946	3.343	0.062

Table F. Results of an AICc test for model selection investigating parental effects on chick baseline CORT. Models were selected from a set of candidate models, with all models with ΔAICc < 2 regarded as indistinguishable. All chicks were about 25 days old (24-29 days). CORT nestlings, n = 11. SHAM nestlings, n = 10.

Response variable	Candidate model	K	AICc	Delta AICc	AICcWt
Chick baseline CORT	Null model	2	72.290	0.000	0.282
	Parental baseline CORT	3	73.262	0.971	0.173
	Sibling	3	73.677	1.387	0.141
	Chick age	3	73.971	1.681	0.122
	Chick sex	3	74.195	1.901	0.110
	Parental CORT treatment	3	74.264	1.974	0.105
	Parental BCI	3	75.084	2.794	0.070

Table G. Results of an AICc test for model selection investigating parental effects on chick BCI. Models were selected from a set of candidate models, with all models with ΔAICc < 2 regarded as indistinguishable. All chicks were about 25 days old (24-29 days). CORT nestlings, n = 11. SHAM nestlings, n = 10.

Response variable	Candidate model	K	AICc	Delta AICc	AICcWt
Chick BCI	Parental CORT treatment	3	195.593	0.000	0.652
	Sibling	3	199.520	3.927	0.092
	Null model	2	199.685	4.093	0.084
	Parental baseline CORT	3	200.072	4.480	0.070
	Chick sex	3	200.693	5.100	0.051
	Chick age	3	201.715	6.123	0.031
	Parental BCI	3	202.471	6.8780	0.021

AICc tests with chicks of 2011 & 2012

Table H. Results of an AICc test for model selection investigating parental effects on chick H:L ratio. Models were selected from a set of candidate models, with all models with ΔAICc < 2 regarded as indistinguishable. All chicks were about 25 days old (24-29 days). CORT nestlings, n = 11. SHAM nestlings, n = 10. 2012 nestlings, n = 23.

Response variable	Candidate model	K	AICc	Delta AICc	AICcWt
H:L ratio	Parental CORT treatment	3	-32.267	0.000	0.642
	Chick sex	3	-29.306	2.961	0.146
	Chick age	3	-28.225	4.042	0.085
	Null model	2	-27.844	4.423	0.070
	Parental BCI	3	-26.114	6.153	0.030
	Sibling	3	-25.787	6.481	0.025
	Parental baseline CORT	3	-21.167	11.100	0.003

Table I. Results of an AICc test for model selection investigating parental effects on chick lysis score. Models were selected from a set of candidate models, with all models with ΔAICc < 2 regarded as indistinguishable. All chicks were about 25 days old (24-29 days). CORT nestlings, n = 11. SHAM nestlings, n = 10. 2012 nestlings, n = 23.

Response variable	Candidate model	K	AICc	Delta AICc	AICcWt
Lysis score	Group	3	116.837	0.000	0.644
	Parental baseline	3	118.903	2.066	0.229
	Parental CORT treatment				
	Chick age	3	121.310	4.473	0.069
	Chick sex	3	122.544	5.707	0.037
	Null model	2	125.041	8.204	0.011
	Parental BCI	3	126.305	9.469	0.001
	Sibling	3	126.656	9.819	0.001

Table J. Results of an AICc test for model selection investigating parental effects on chick baseline CORT. Models were selected from a set of candidate models, with all models with ΔAICc < 2 regarded as indistinguishable. All chicks were about 25 days old (24-29 days). CORT nestlings, n = 11. SHAM nestlings, n = 10. 2012 nestlings, n = 23.

Response variable	Candidate model	K	AICc	Delta AICc	AICcWt
Chick baseline CORT	Parental baseline CORT	3	152.391	0.000	0.978
	Parental CORT treatment	3	161.160	8.769	0.012
	Null model	2	163.485	11.094	0.004
	Chick age	3	164.514	12.123	0.002
	Parental BCI	3	165.430	13.039	0.001
	Chick sex	3	165.580	13.189	0.001
	Sibling	3	165.800	13.409	0.001

Table K. Results of an AICc test for model selection investigating parental effects on chick BCI. Models were selected from a set of candidate models, with all models with ΔAICc < 2 regarded as indistinguishable. All chicks were about 25 days old (24-29 days). CORT nestlings, n = 11. SHAM nestlings, n = 10. 2012 nestlings, n = 23.

Response variable	Candidate model	K	AICc	Delta AICc	AICcWt
Chick BCI	Parental baseline CORT	3	406.191	0.000	1.000
	Sibling	3	432.470	26.278	0.000
	Null model	2	443.244	37.053	0.000
	Chick sex	3	444.329	38.138	0.000
	Chick age	3	444.784	38.593	0.000
	Parental CORT treatment	3	444.904	38.713	0.000
	Parental BCI	3	445.099	38.910	0.000